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## Cuticular hydrocarbons of larva and pupa of Reverdin's blue, *Lycaeides argyrogynomon* (Lycaenidae) and its tending ants

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**Abstract** The immature of Reverdin's blue butterfly, *Lycaeides argyrogynomon* (Lycaenidae), exhibits facultative symbiosis with several ant species; *Camponotus japonicus*, *Formica japonica*, *F. yessensis*, *Pristomyrmex punctatus*, and two other species are its tending ants observed in Yamanashi Pref. In our attempt to clarify key factors regulating the symbiotic association between this myrmecophilous butterfly and ants, we examined the chemical compositions of cuticular hydrocarbons (CHCs) of third- and fourth-instar larvae and pupae of the butterfly, together with those of the above ant species. Larvae, irrespective of their instars and attendant ant species, showed a consistent CHC profile, in which *n*-alkanes, especially C27 and C29, were predominant, with methyl-branched alkanes being minor components. Pupae also possessed almost the same set of CHCs as did larvae. In contrast, the four ant species exhibited distinct and species-specific CHC profiles. The CHCs of *C. japonicus* and *F. japonica* consisted mainly of *n*-alkanes and alkenes of C27 and C29, while those of *F. yessensis* and *P. punctatus* contained *n*-alkanes of C25, C27, C29, and C31. The results strongly suggest that the protection of *L. argyrogynomon* from aggression by ant workers is not due to concealment by chemical camouflage based on CHCs, as is known in some *Maculinea* butterflies.

**Key words** Myrmecophily, *Lycaeides argyrogynomon*, cuticular hydrocarbon, tending ant, facultative symbiosis.

### Introduction

The estimated 6,000 species of Lycaenidae account for about one third of all Papilioidea. The majority of lycaenid butterflies are myrmecophilous and their ant-associations can be categorized principally into two types: obligate symbiosis with specific ant species and facultative symbiosis with multiple ant species (Fiedler, 1998; Pierce *et al.*, 2002). The Reverdin's blue, *Lycaeides argyrogynomon* (Lycaenidae), is a multivoltine butterfly inhabiting midstream river beds and volcanic grasslands (Fukuda *et al.*, 1992). This lycaenid exhibits facultative symbiosis with several ant species in the immature stage. Larvae feed on the leaves of *Indigofera pseudo-tinctoria* (Leguminosae) and provide the worker ants with nutritious secretions from dorsal nectary organs in exchange for protection against predators and parasites (Fig. 1A), while pupae tended by ants are often observed inside the ant nest hole or on the host plant (Fig. 1B) (Watanabe and Hagiwara, 2009). The species of tending ants vary temporally and spatially: *e. g.* *Camponotus japonicus* and *Formica japonica* (subfamily Formicinae) are the most frequent species in Yamanashi Pref., while *F. yessensis* (Formicinae), *Pristomyrmex punctatus* (Myrmicinae) and *Lasius japonicus* (Formicinae) are opportunistic and regional species (Watanabe, 2006 & unpublished data). Recently a severe decline of *L. argyrogynomon* population in Japan has been reported, and it has been included as a vulnerable species in the red data lists of the Ministry of the environment. For conser-

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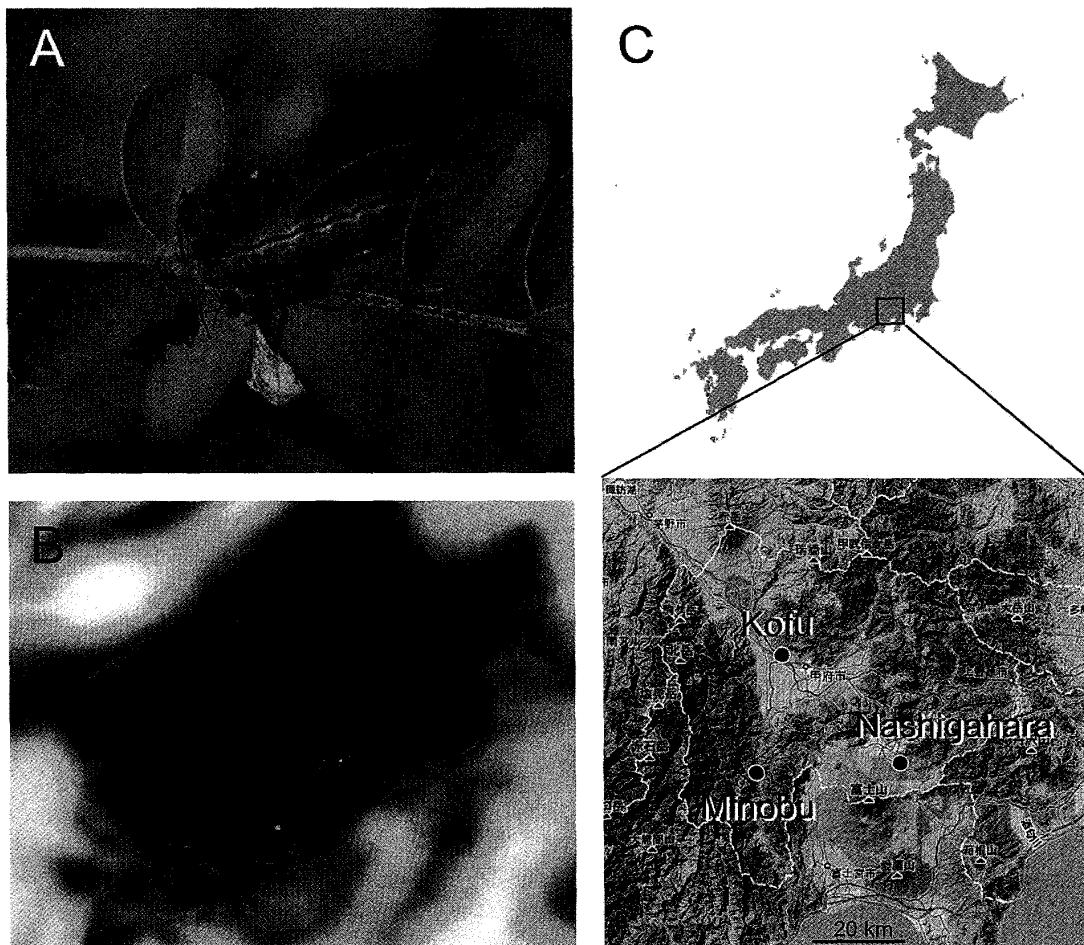


Fig. 1. A. *L. argyrogynomon* larva tended by *F. japonica* on the host plant (photo by M. Watanabe). B. *L. argyrogynomon* pupa tended by *C. japonicus* inside the ant nest hole (photo by M. Watanabe). C. Collection sites of immatures of *L. argyrogynomon* and its tending ants in Yamanashi Pref.

vation of this myrmecophilous butterfly, it is important to discover the key factors regulating its ant-association.

The juveniles of lycaenid butterflies use complex chemical and acoustical signals to manipulate ants (Pierce *et al.*, 2002). Among these signals, cuticular hydrocarbons (CHCs) are considered to be one of the crucial cues in the regulation of lycaenid-ant associations. For example, several lycaenid butterflies of the genus *Maculinea* and *Niphanda*, which are well known for their obligate symbiosis inside ant nests, chemically mimic the CHC profiles of the ant partners to protect them from worker aggression (Akino *et al.*, 1999; Nash *et al.*, 2008; Hojo *et al.*, 2009). On the other hand, there has been little attempt to examine whether lycaenid species with facultative symbiosis, as well as those with obligate symbiosis, mimic the CHCs of host ants. We describe here the CHC profiles of *L. argyrogynomon* in the immature stage, together with those of four species of its tending ants, and discuss the possibility that this lycaenid butterfly employs chemical camouflage in its ant-association.

## Materials and methods

### Insects

Immatures of *L. argyrogynomon*, 17 fourth-instar larvae, 9 third-instar larvae, and 7 pupae, were collected from Nashigahara, Kofu, and Minobu populations in Yamanashi Pref. in Aug. and Sep. 2007 and in Sep. 2008 (Fig. 1C). Among the fourth instars, 4, 3, 4, and 1 individuals were tended by *C. japonicus*, *F. japonica*, *F. yessensis*, and *P. punctatus*, respectively. Among the third instars, 6, 1, and 1 larvae were tended by *C. japonicus*, *F. japonica*, and *F. yessensis*, respectively. All pupae were tended by *F. japonica*. Tending ants were collected from the same populations of *L. argyrogynomon*. *C. japonicus* and *F. japonica* were originated from Nashigahara, Kofu and Minobu, while *F. yessensis* was captured in Nashigahara and *P. punctatus* was collected in Kofu, respectively.

### Extraction

Live larvae and pupae were individually soaked in 1 ml of purified (twice distilled) dichloromethane for 3 min. Tending ants were frozen to death at -20°C and were soaked individually or together with 2–4 individuals in 500 µl or 1 ml of purified dichloromethane for 3 min. The resulting extracts were filtrated and concentrated to 50 or 100 µl under a nitrogen stream at 10°C. The concentrated extracts were stored at -20°C until analysis.

### Chemical analyses of cuticular hydrocarbons

The concentrated extracts were examined by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed at 70 eV using a Shimadzu QP5000 mass spectrometer coupled with a Shimadzu GC-17A gas chromatograph equipped with a J&W Scientific DB-1 fused-silica capillary column (0.25 mm ID × 15 m, 0.25 µm film thickness), with a temperature program of 50°C (held initially for 1 min) to 320°C (held for 5 min) at 10°C/min. The interface temperature was 320°C. Samples were injected splitless at 320°C using He as the carrier gas (1.9 ml/min). To calculate equivalents of chain length (ECL) for CHC components, a series of authentic *n*-alkane samples commercially purchased (Tokyo Chemical Industry and Sigma-Aldrich) were analyzed in the same analytical conditions for the extracts. Identification of the *n*-alkane components was based on comparison of retention data and mass spectra with those authentic samples, while a tentative identification of other hydrocarbon components was based on comparison of ECL data and mass spectra which has been previously reported (Akino *et al.*, 2002; Akino, 2006; Tanigaki *et al.*, 2007). Each CHC component in the extracts of the immatures of *L. argyrogynomon* was quantified using an authentic *n*-alkane with the same carbon number in the principal chain.

## Results

### Larval and pupal CHCs

Larval CHC profile, irrespective of their instars, tending ant species, and origin of populations, was qualitatively consistent and composed mainly of *n*-alkanes of C25 to C33, predominant components of which were *n*-heptacosane (C27) and *n*-nonacosane (C29) (Fig. 2, Table 1). In addition, several methyl-branched alkanes were present as minor CHC components. In terms of CHC amounts, the fourth instar had three to four times more than the third instar. Although pupae showed inter-individual differences in the quantities of components, the CHC profile was qualitatively similar to that of the larvae, in which the major CHCs were *n*-heptacosane (C27) and *n*-nonacosane (C29). Highly volatile components were never detected in either larval or pupal extracts.

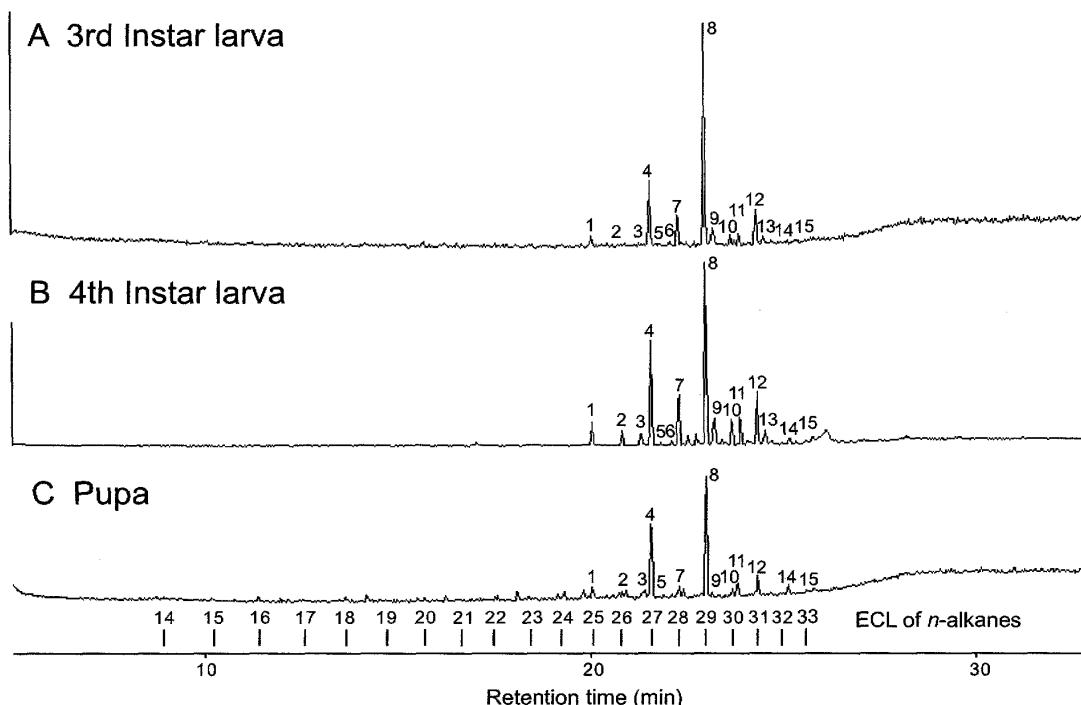


Fig. 2. Typical total ion chromatograms of the dichloromethane extracts of *L. argyrogynomon* larvae (A and B) and pupa (C). Peak numbers correlate to those in Table 1.

Table 1. Chemical composition of cuticular hydrocarbon components of larvae and pupae of *L. argyrogynomon*.

Peak No.	RT(min)	ECL	Compound	Larvae		Pupae	
				4th Instar (N=17)	3rd Instar (N=9)	(N=7)	Amount per individual (mean±SD) [ng]
1	20.14	25.00	<i>n</i> -Pentacosane	77 ± 45	29 ± 15	24 ± 7	
2	20.85	26.00	<i>n</i> -Hexacosane	55 ± 39	19 ± 10	7 ± 5	
3	21.33	26.64	4-Methylhexacosane <sup>a</sup>	26 ± 17	11 ± 6	10 ± 15	
4	21.60	27.00	<i>n</i> -Heptacosane	437 ± 254	148 ± 72	71 ± 35	
5	21.85	27.34	13/11/9-Methylheptacosane <sup>a</sup>	13 ± 9	5 ± 4	10 ± 11	
6	22.14	27.74	7,15-Dimethylheptacosane <sup>a</sup>	24 ± 19	9 ± 4	N.D. <sup>b</sup>	
7	22.33	28.00	<i>n</i> -Octacosane	231 ± 175	45 ± 16	17 ± 11	
8	23.04	29.00	<i>n</i> -Nonacosane	1116 ± 892	297 ± 116	272 ± 201	
9	23.25	29.31	15/13/11-Methylnonacosane <sup>a</sup>	80 ± 70	15 ± 11	N.D.	
10	23.71	30.00	<i>n</i> -Triacontane	170 ± 152	25 ± 12	12 ± 7	
11	23.92	30.32	14/12-Methyltriacontane <sup>a</sup>	105 ± 89	23 ± 17	12 ± 13	
12	24.37	31.00	<i>n</i> -Hentriacontane	309 ± 269	45 ± 26	55 ± 41	
13	24.56	31.31	15/13/11-Methylhentriacontane <sup>a</sup>	51 ± 48	7 ± 4	N.D.	
14	25.18	32.31	14/12-Methyldotriacontane <sup>a</sup>	15 ± 15	5 ± 5	57 ± 87	
15	25.61	33.00	<i>n</i> -Tritriacontane	28 ± 18	7 ± 6	5 ± 7	

<sup>a</sup>Tentative identification by comparison of ECL data previously reported.

<sup>b</sup>Not detected.

### CHC profiles of tending ants

The four species of tending ants for *L. argyrogynomon* showed distinct and species-specific chemical compositions of CHCs (Fig. 3; Table 2). *C. japonicus* exhibited a constant CHC profile among three different colonies. The chemical composition was similar to that of colony 3 described in Ozaki *et al.* (2005), where 7- and 9-alkenes of C27 and C29 were predominant, while *n*-alkanes of C23 to C29 and methyl-branched alkanes were also detected.

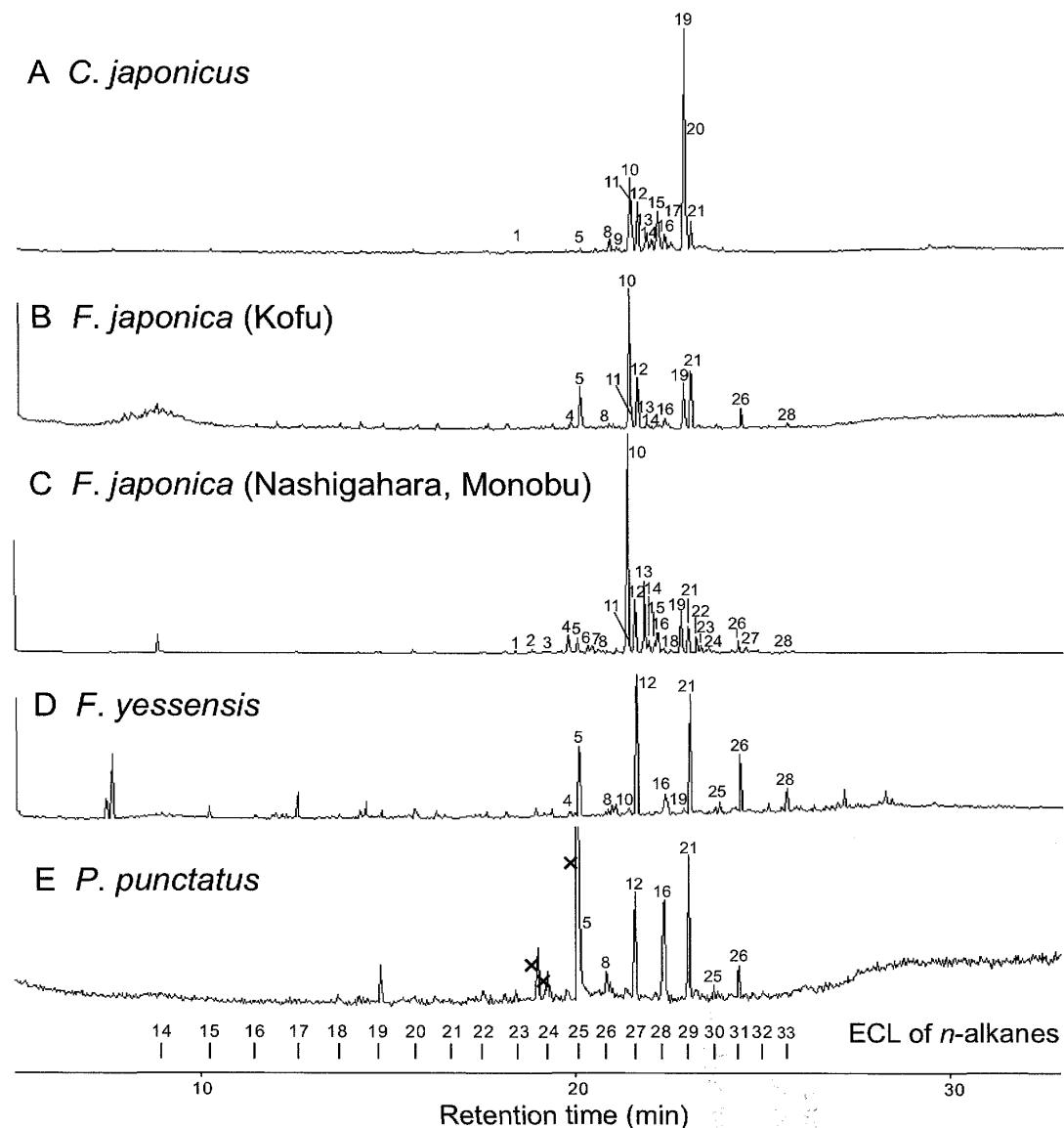


Fig. 3. Typical total ion chromatograms of the dichloromethane extracts of four ant species, *C. japonicus* (A), *F. japonica* (B and C), *F. yessensis* (D), and *P. punctatus* (E). Peak numbers correlate to those in Table 2. The peaks with an X mark in chromatogram E are derived from artifacts.

The CHC profiles of *F. japonica* were qualitatively different between the Kofu colony and the Nashigahara and Minobu colonies. Major CHCs of the Kofu colony were 9-alkenes of C27 and C29, and *n*-alkanes of C25, C27, and C29, while those of the Nashigahara and Minobu colonies were 9-heptacosene, *n*-heptacosane, and 13-methylheptacosane. In terms of CHC profile, the Kofu colony and the Nashigahara and Minobu colonies were similar to types 2 and 3 of *F. japonica* reported in Akino *et al.* (2002), respectively. The CHCs of *F. yessensis* and *P. punctatus* mainly consisted of *n*-alkanes, especially C25, C27, C29, and C31, and contained few alkenes and methyl-branched alkanes.

## Discussion

The larval CHCs of *L. argyrognomon* included a significant proportion of *n*-alkanes (nearly

Table 2. Expected cuticular hydrocarbon components of ant species tending larvae and pupae of *L. argyrogynon*.

Peak No.	RT(min)	ECL	Expected compound	<i>C. japonicus</i>			<i>F. japonica</i>	<i>F. yessensis</i>	<i>P. punctatus</i>
				N, K, M <sup>a</sup>	K	N, M	N	K	K
1	18.39	23.00	<i>n</i> -Tricosane	+			+		
2	18.84	23.41	9-Methyltricosane		+	+	+	*	***
3	19.48	24.00	<i>n</i> -Tetracosane			+	+	+	
4	19.79	24.55	9-Pentacosane		+	**			
5	20.04	25.00	<i>n</i> -Pentacosane	+			+	*	
6	20.33	25.37	13/11-Methylpentacosane				+		
7	20.44	25.51	5-Methylpentacosane				+	*	
8	20.82	26.00	<i>n</i> -Hexacosane	+			+		
9	21.01	26.25	5,7,12/7,9,12-Trimethylpentacosane	+			+		
10	21.36	26.71	9-Heptacosene	**			***	+	
11	21.43	26.80	7-Heptacosene	**	+		**		***
12	21.58	27.00	<i>n</i> -Heptacosane	*	**		**		***
13	21.83	27.35	13-Methylheptacosane	*			**		
14	21.95	27.51	5-Methylheptacosane	*	+		+		
15	22.11	27.74	7,15-Dimethylheptacosane	**			+		
16	22.30	28.00	<i>n</i> -Octacosane	+			*		
17	22.48	28.26	5,7,12-Trimethylheptacosane	*			+		
18	22.53	28.33	14-Methyloctacosane				+		
19	22.82	28.74	9-Nonacosene	***			*		
20	22.89	28.84	7-Nonacosene	**			**		
21	23.00	29.00	<i>n</i> -Nonacosane	*			*		***
22	23.23	29.32	15/13/11-Methylnonacosane				+		
23	23.35	29.53	5-Methylnonacosane				+		
24	23.56	29.84	Dimethylnonacosane				+		
25	23.71	30.00	<i>n</i> -Triacontane				+		
26	24.33	31.00	<i>n</i> -Hentriacontane	*			+		**
27	24.53	31.32	15/13/11-Methylhentriacontane				+		
28	25.58	33.00	<i>n</i> -Tritriacontane	+			+		*

<sup>a</sup> N: Nashigahara colony; K: Kofu colony; M: Minobu colony.<sup>b</sup> \*\*\*: >50% of relative peak intensity; \*\*: <50% >20% of relative peak intensity; \*: <20% >10% of relative peak intensity;

+: &lt;10% of relative peak intensity.

90%) and a small proportion of methyl-branched alkanes. The predominant components were *n*-heptacosane (C27) and *n*-nonacosane (C29). In contrast, few alkene components were detected. Pupae also possessed the same set of CHCs as did larvae. Since collected wild individuals were employed for chemical analyses, the CHC profiles had been expected to show large inter-individual difference depending on larval instar, tending ant species, and origin of population. Nevertheless, the larval and pupal CHCs showed qualitative consistency and similarity each with other. These results are most likely to reflect the innate traits of CHCs in the immature stage of *L. argyrognomon*.

On the other hand, the four species of the tending ants exhibited species-specific chemical compositions of CHCs. The CHCs of *C. japonicus* and *F. japonica* consisted mainly of *n*-alkanes and alkenes, while those of *F. yessensis* and *P. punctatus* were composed predominantly of *n*-alkanes and were relatively similar to that of *L. argyrognomon*. These results strongly suggest that *L. argyrognomon* does not employ chemical camouflage based on the CHCs of its tending ant species, as is known in *Maculinea rebeli*, *M. alcon*, and *Niphanda fusca* (Akino *et al.*, 1999; Nash *et al.*, 2008; Hojo *et al.*, 2009). Facultatively symbiotic lycaenid butterflies, including *L. argyrognomon*, might employ different strategies from obligately symbiotic species to regulate their ant-associations.

Worker ants use CHCs as the crucial cues to recognize their nest mates: once certain workers encounter ants with different CHC profiles, they conduct aggression behavior such as biting and spraying acidic secretions. Recent studies have revealed the key factors for nest-mate recognition in the CHC profiles of several ant species. *Formica exsecta* (Formicinae) discriminate between nest mates and non-nest mates on the basis of the abundance of (Z)-9-alkene part of the CHC profile (Martin *et al.*, 2008). Other ant species conduct nest-mate recognition based on the relative abundance of CHCs among the different structural classes, *e.g.* *F. japonica* uses *n*-alkane and (Z)-9-alkene parts of the CHC profile (Akino *et al.*, 2004), while *Linepithema humile* (Dolichoderinae) and *Aphaenogaster cockerelli* (Myrmicinae) use at least two parts of *n*-alkanes, methyl-branched alkanes, and alkenes (Greene and Gordon, 2007). The CHC profile of *L. argyrognomon*, such as the dominance of *n*-alkanes and the absence of alkene, might contribute something to concealing the immatures from recognition by ant workers and to protecting them from aggression. Further investigations are necessary to reveal potential functions of the CHCs of *L. argyrognomon* in its facultative symbiosis with several ant species.

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## 摘要

ミヤマシジミ幼虫・蛹とその随伴アリの体表炭化水素(大村 尚・渡邊通人・本田計一)

ミヤマシジミの幼虫や蛹は数種のアリと任意的な共生関係を築いており、山梨県ではクロヤマアリ、クロオオアリ、エゾアカヤマアリ、アミメアリなどが随伴アリとして観察されている。我々はミヤマシジミとアリとの共生関係を制御する要因を明らかにすることを目的とし、3齢・4齢幼虫や蛹の体表炭化水素(CHC)の化学組成について、上記随伴アリの組成と共に調べた。幼虫は、齢や随伴アリの種類に関わらず一定のCHCプロファイルを示した。その主成分はC27, C29を主成分とする直鎖アルカンであり、副成分は分枝アルカンであった。蛹も幼虫と同じCHCを持っていた。一方、4種の随伴アリは識別可能で種特異的なCHCプロファイルを示した。クロオオアリとクロヤマアリのCHCはC27, C29の直鎖アルカンとアルケンを主成分にしており、エゾアカヤマアリとアミメアリのCHCはC25, C27, C29, C31の直鎖アルカンであった。以上の結果からミヤマシジミは、数種のゴマシジミ族が利用することで知られるCHCの化学擬態とは異なる機構を用いて働きアリの攻撃を回避すると考えられる。

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